RESEARCH

Assessment of Genetic Diversity among Nondormant and Semidormant Alfalfa Populations Using Sequence-Related Amplified Polymorphisms

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ABSTRACT

Synthetic populations of alfalfa (*Medicago sativa* L.) consist of genetically heterogeneous mixtures of plants, which may complicate use of molecular markers for examining genetic relationships among populations. Molecular marker techniques combined with bulked DNA from multiple plants provides perhaps the best combination of accuracy and high throughput for characterization and differentiation of alfalfa populations. Using 20-plant bulked DNA templates we assessed how sequence-related amplified polymorphisms (SRAPs) would estimate genetic similarity among 29 nondormant and semidormant alfalfa populations representing diverse genetic backgrounds and the nine historically recognized U.S. germplasm sources. Cluster analysis based on estimates of genetic similarity showed separation of populations in fall dormancy classes 6 to 8 (semidormant) from fall dormancy classes 9 to 11 (nondormant) and in many examples, DNA bulks of the same population clustered together. A single SRAP marker was identified that was present only in bulks of fall dormancy classes 6 and 7, and another marker was identified that was detected only in bulks of fall dormancy class 8. This study demonstrates that the amplification of SRAP markers from DNA extracted from bulked plant samples is an efficient method for estimating genetic similarity both within and between a large numbers of populations. The SRAPs may also be useful for indirectly classifying relative fall dormancies of uncharacterized populations.

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Abbreviations: FDC, fall dormancy class; NAAIC, North American Alfalfa Improvement Conference; NAMLVRB, National Alfalfa and Miscellaneous Legume Variety Review Board; PCA, principal component analysis; PCR, polymerase chain reaction; PI, plant introduction; SRAP, sequence-related amplified polymorphism.

Many agronomic adaptations have been incorporated into modern cultivated alfalfa (*Medicago sativa* L.) to make it the successful forage crop grown on nearly 9 million ha in the United States today (National Agricultural Statistical Service, 2006). Fall dormancy is a critical trait for successful cultivation of alfalfa in specific climatic zones. Alfalfa cultivars can be broadly grouped as dormant, semidormant, or nondormant and specifically categorized by fall dormancy class (FDC). Phenotypically, dormant alfalfas (FDC 1–4) are characterized by reduced fall shoot growth, slow regrowth after harvest, and decumbent habit while nondormant types (FDC 8–11) have rapid fall shoot growth, rapid regrowth after harvesting and upright habit (Lehman et al., 1987). As the term implies, semidormant alfalfas (FDC 5–7) exhibit intermediate traits of both dormant and nondormant types.

Barnes et al. (1977) suggested that North American alfalfa cultivars are predominately derived from nine distinct sources of germplasm. These germplasm sources are *M. falcata*, *M. sativa*

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'Ladak', *M. varia*, a naturally occurring hybrid between *M. sativa* and *M. falcata*, *M. sativa* 'Chilean', *M. sativa* 'Flemish', *M. sativa* 'Turkistan', *M. sativa* 'Peruvian', *M. sativa* 'Indian', and *M. sativa* 'African'. These germplasm sources were classified primarily based on geographic origin, flower color, and fall dormancy characteristics (Barnes et al., 1977). *Medicago falcata*, *M. varia*, Ladak, and Turkistan are considered to be sources of fall dormancy in commercial cultivars (Barnes et al., 1977). The Indian, African, Chilean, and Peruvian germplasm sources are considered to primarily contribute to nonfall dormant cultivars (Barnes et al., 1977; Lehman et al., 1987).

Several studies have demonstrated correlations between reduced fall dormancy and winter survival (Smith, 1958, 1961; Stout, 1985). Although genetic analysis suggests that these two traits may be inherited independently (Brouwer et al., 2000; Brummer et al., 2000), nondormant alfalfa cultivars have been observed to have significantly lower winter survival than more dormant cultivars when grown under identical methods of harvest management in locations having severe winters (Sheaffer et al., 1992). In addition, selection for increased fall dormancy in the nondormant cultivar CUF101 (Lehman et al., 1983) resulted in improved winter survival (Cunningham et al., 1998).

Alfalfa cultivars are placed in a FDC based on a protocol established by the North American Alfalfa Improvement Conference (NAAIC), which relies on regrowth measurements in fall, multiple years of data, and comparison to cultivars of known FDC to determine placement of a cultivar into 1 of 11 classes (Teuber et al., 1998). The protocol, while widely accepted and successful in FDC determination, is time and labor intensive. The identification of molecular markers that are tightly linked to traits that are difficult to accurately evaluate without multiple years of data, such as fall dormancy, could accelerate the process of alfalfa cultivar development by allowing for the direct selection of seedlings with desired genotypes. However, few examples exist in the literature describing the identification of molecular markers linked specific phenotypes in tetraploid alfalfa (Brouwer et al., 2000; Musial et al., 2005; Robins et al., 2007a,b). The paucity of useful markers for alfalfa breeding is likely due to difficulties associated with identifying marker linkages in tetraploid genomes and the inability to produce inbred lines because of inbreeding depression.

Most studies using molecular markers in alfalfa have focused on examining genetic relationships between different populations. Commercial alfalfa cultivars are predominately synthetic populations resulting from multiple cycles of random mating among selected parental plants and their progeny (Busbice et al., 1972). As a consequence of repeated cycles of random mating, cultivars consist of genetically heterogeneous mixtures of genotypes. The genetic diversity present between plants in a given cultivar creates challenges for developing marker experiments that adequately examine a sufficient number of plants in a cultivar to sample the genetic variation present within the cultivar, while permitting the examination of a large number of different cultivars.

Several efforts have attempted to examine genetic relationships between alfalfa populations by using molecular markers to detect polymorphisms in DNA extracted from bulked plant samples. Kidwell et al. (1994) separated *M. falcata* (Bingham, 1993) and Peruvian germplasm from the other seven sources of *M. sativa* germplasm by detecting restriction fragment length polymorphisms (RFLPs) (Beckmann and Soller, 1983) between four and six plant bulk DNA extracts. Yu and Pauls (1993) demonstrated that randomly amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990) could distinguish alfalfa populations and cultivars based on the amplification of bulk DNA collected from 10 plants. Segovia-Lerma et al. (2003) used amplified fragment length polymorphisms (AFLPs) (Vos et al., 1995) and bulk DNA to separate the nine germplasm sources into two main clusters, one containing *M. falcata* and another containing the other eight germplasm sources. Within the cluster containing all sources of germplasm except for *M. falcata* was another subcluster supported by a bootstrap value >50 that contained Ladak, Flemish, and *M. varia* germplasm. Genetic relationships between populations have been estimated using molecular markers and bulked plant DNA for several other crop species, including Kentucky bluegrass (*Poa pratensis* L.) (Johnson et al., 2002), safflower (*Carthamus tinctorius* L.) (Sehgal and Raina, 2005), and crested wheatgrass (*Agropyron cristatum* Gaertn.) (Mellish et al., 2002).

Molecular markers vary in their ability to detect genetic polymorphisms in a timely and cost-effective manner. Sequence related amplified polymorphisms (SRAPs) (Li and Quiros, 2001) are DNA markers amplified by PCR in a single tube using forward and reverse primers with the following sequence motifs: (i) the first 10 to 11 nucleotides at the 5′ end of each primer is a random sequence of nucleotides, similar to primers used to amplify RAPDs; (ii) the sequence CCGG in the forward primer and AATT in the reverse primer, and (iii) three selective nucleotides at the 3′ end of each primer, which is reminiscent of primers used in the secondary PCR to generate AFLPs. The CCGG sequence in the forward primer results in this primer preferentially annealing to exonic sequences, while the AATT domain in the reverse primer causes preferential annealing to introns. Li and Quiros (2001) observed that 60% of the SRAPs amplified and sequenced from *Brassica oleracea* L. shared significant homology to reported gene sequences in GENBANK, suggesting that SRAPs preferentially amplify sequences in open reading frames (ORFs). The SRAPs are evenly distributed among linkage groups and highly reproducible

(Li and Quiros, 2001). The SRAPs have also been shown to provide good distinction of populations of buffalograss (*Buchloe* spp.) (Budak et al., 2004), wheat (*Triticum aestiva* L.) (Fufa et al., 2005), squash (*Cucurbita moschata* L.) (Ferriol et al., 2004) and oilseed rape (*Brassica napus* L.) (Riaz et al., 2001). Vandemark et al. (2006) demonstrated that SRAPs amplified from bulked DNA could separate a small set of public alfalfa cultivars from the nine historical sources of alfalfa germplasm.

Table 1. Alfalfa cultivars and populations included in study with respective fall dormancy class (FDC), and breeding source.

Cultivar/population	Breeding source	FDC
5681	Pioneer Hi-Bred International, Inc.	6
Lobo	ABI Alfalfa, Inc.	6
Tahoe	Forage Genetics International	6
Wilson	Public	6
Amerigraze 701	ABI Alfalfa, Inc.	7
Arriba	ABI Alfalfa, Inc.	7
Dona Ana	Public	7
Dura 765	Cal West Seeds	7
Malone	Public	7
Rio	Great Plains Research Company	7
13R Supreme	ABI Alfalfa, Inc.	8
58N57	Pioneer Hi-Bred International, Inc.	8
Alto	Great Plains Research Company	8
Ameristand 801S	ABI Alfalfa, Inc.	8
5959	Pioneer Hi-Bred International, Inc.	8
DK 180ML	Forage Genetics International	8
DK 189	Cal West Seeds	8
Magna 801FQ	Dairyland Seeds Co., Inc.	8
RioGrande	Great Plains Research Company	8
5929	Pioneer Hi-Bred International, Inc.	9
59N49	Pioneer Hi-Bred International, Inc.	9
Beacon	Forage Genetics International	9
CUF 101	Public	9
DK 194	Cal West Seeds	9
Salado	ABI Alfalfa, Inc. (America's Alfalfa)/Novartis	9
Sedona	Forage Genetics International	10
UC 1887	Public	10
WL 711	Forage Genetics International	10
UC 1465	Public	11
African (PI 536539)	Melton et al. (1990)	Nondormant
Chilean (PI 536534)	Melton et al. (1990)	Nondormant
Flemish (PI 536538)	Melton et al. (1990)	Semidormant
Indian (PI 536536)	Melton et al. (1990)	Nondormant
Ladak (PI 536532)	Melton et al. (1990)	Dormant
M. falcata (PI 530333, WISFAL)	Bingham (1993)	Dormant
M. varia (PI 536533)	Melton et al. (1990)	Semidormant
Peruvian (PI 536535)	Melton et al. (1990)	Nondormant
Turkistan (PI 536537)	Melton et al. (1990)	Semidormant/ dormant

The ability to determine genetic relationships between alfalfa populations can confer several benefits to breeding programs, including minimizing redundancies among populations and clones maintained as parental materials. An improved understanding of the genetic structure within specific populations will also provide essential knowledge of the effects of multiple cycles of random mating on the genetic heterogeneity of synthetic cultivars. Ultimately identifying markers closely associated with traits that require laborious tests and multiple years of data, such as fall dormancy, would greatly simplify selection of plants with desirable genotypes. Previous efforts to characterize North American alfalfa populations with molecular markers have collectively only examined the nine original sources of germplasm and a small subset of cultivars (Bauchan et al., 2003; Kidwell et al., 1994; Segovia-Lerma et al., 2003; Yu and Pauls, 1993; Vandemark et al., 2006). The objectives of this study were (i) to use SRAPs and bulked DNA to estimate genetic similarities among the nine populations representing the original sources of *Medicago* germplasm in North America and 29 semidormant and nondormant alfalfa cultivars, (ii) to survey genetic variation within these alfalfa populations using SRAPs, and (iii) to examine the ability of SRAPs to differentiate among cultivars of different FDCs.

MATERIALS AND METHODS

Plant Populations

Thirty-eight alfalfa populations representing 29 cultivars and the nine historically recognized U.S. germplasm sources were selected for the present study (Table 1). The selected cultivars were developed by various public breeding programs and several companies that are responsible for the majority of the alfalfa cultivars grown in North America. All included cultivars had reported fall dormancy classes of FDC 6 to 11.

DNA Extraction

Each bulk DNA sample was derived from 20, 3- to 4-d-old seedlings germinated on sterilized filter paper. Five bulked DNA samples were generated for each population (cultivar or PI). DNA was extracted from the 20 seedlings using FastDNA Kits and the FastPrep Instrument (Qbiogene, Inc., Irvine, CA) per the manufacturer's protocol. Bulk DNA samples were quantified with a fluorometer (TD-700, Turner Designs Inc., Sunnyvale, CA) and adjusted to 10 ng μL^{-1}.

Sequence-Related Amplified Polymorphism Reactions

Seven SRAP primer pair combinations (forward primer plus reverse primer) were selected for the study based on previously observed ability to generate polymorphisms in alfalfa populations. These SRAP primer pairs were F7/em5, F11/R15, F12/R9, me2/R9, me4/R14, me4/em2, and F9/R9 (Table 2). The SRAPs were amplified from 25 μL reaction volumes using con-

ditions and thermocycling profiles previously described (Vandemark et al., 2006) for detecting SRAPs from bulked alfalfa DNA. All PCR reactions were completed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). Fifteen μL of PCR product per reaction were resolved by gel electrophoresis at 100 V for 5 h using Criterion precast 15% PAGE-TBE gels (Bio-Rad, Hercules, CA). Gels were stained in 0.01% (w/v) ethidium bromide solution for 30 min and amplicons were visualized with UV light. The reproducibility of SRAP reactions was evaluated by taking a subset of bulk DNA samples (Indian bulks 1–3, *M. varia* bulks 1,2, and 5, Beacon bulks 1–3, and Ameristand 801S bulks 1–3) and performing replicate SRAP reactions with the primer pairs F7/em5, F9/R9, and F11/R15 using two different GeneAmp PCR System 9700 thermocyclers. Both replicates of each sample/primer pair combination were resolved in adjacent wells of 15% PAGE-TBE gels as described above.

Data Analysis

Gel images were captured and scored using TotalLab TL120 v.2006 (Nonlinear Dynamics LTD, Newcastle on Tyne, UK). Genetic similarities (G_s) (Nei and Li, 1979) were derived from the presence/absence data as follows: $G_s = 2N_{AB}/(2N_{AB} + N_A + N_B)$, where N_{AB} = number of shared bands present in samples A and B, N_A = number bands present in Sample A, and N_B = number bands present in Sample B.

An unweighted pair-group method using arithmetic averages (UPGMA) cluster analysis was conducted to generate a dendrogram representing genetic similarity based on G_s values of 20-plant bulk DNA samples. Calculations of G_s, cluster analysis and representation were conducted in NTSyspc version 2.20e (Rohlf, 2002). Bootstrap support for clusters was conducted in FreeTree (http://www.natur.cuni.cz/~flegr/freetree.htm) using 1000 permuted datasets (Pavlicek et al., 1999). Principal component analysis (PCA) was conducted using the PRINCOMP procedure in SAS (SAS Institute, Cary, NC) and the broken stick model (MacArthur, 1957) was used to determine the significance of principle components.

RESULTS

Amplification of DNA Bulks with Sequence-Related Amplified Polymorphism Markers

Successful amplification occurred in all DNA bulks and all primer combinations except for a single bulk of Flemish (Bulk 1), which was excluded from the analysis. Replicated reactions with a subset of bulks and primer pairs indicted that the SRAP amplifications were completely reproducible using two different GeneAmp PCR System 9700 thermocyclers (Applied Biosystems, Foster City, CA) (data not shown). The seven primer pairs generated 188 markers ranging in frequency among bulks from 100.0 to 0.5% (Fig. 1). The average number of markers per primer pair was 27. Only six markers (3.9%) were monomorphic (i.e., present in all bulks) and 32 markers were present at <10% in the 189 bulks included in the study. The highest numbers of markers were present in the ≤10% and ≥90% frequency classes (Fig. 1). No cultivar or population-specific amplicons (i.e., present only in all bulks of a single population) were detected. A 105 bp SRAP marker amplified by primer pair me 4/em 2 was detected that was present only in bulked DNAs of FDC 6 and 7. Similarly, a 290 bp SRAP marker amplified by primer pair F 11/R 15 was identified that was present in only in FDC 8 bulks.

Table 2. Summary of forward and reverse primers used for sequence-related amplified polymorphism (SRAP) analyses.

Forward primers†	Reverse primers†
me2, 5′-TGAGTCCAAACCGGAGC-3′	em2, 5′-GACTGCGTACGAATTTGC-3′
me4, 5′-TGAGTCCAAACCGGACC-3′	em5, 5′-GACTGCGTACGAATTAAC-3′
F7, 5′-GTAGCACAAGCCGGAGC-3′	R9, 5′-GACACCGTACGAATTTGA-3′
F9, 5′-GTAGCACAAGCCGGACC-3′	R14, 5′-CGCACGTCCGTAATTAAC-3′
F11, 5′-CGAATCTTAGCCGGATA-3′	R15, 5′-CGCACGTCCGTAATTCCA-3′
F12, 5′-CGAATCTTAGCCGGAGC-3′	

†Primers me2, me4, em2, em5 from Li and Quiros (2001).

Estimating Genetic Similarities among Bulks within Populations

Mean genetic similarities determined based on the results of SRAP markers are presented in Table 3 for all 703 pairwise combinations between populations. Marker polymorphism present within a cultivar or population was assessed by calculating the mean G_s among bulks within

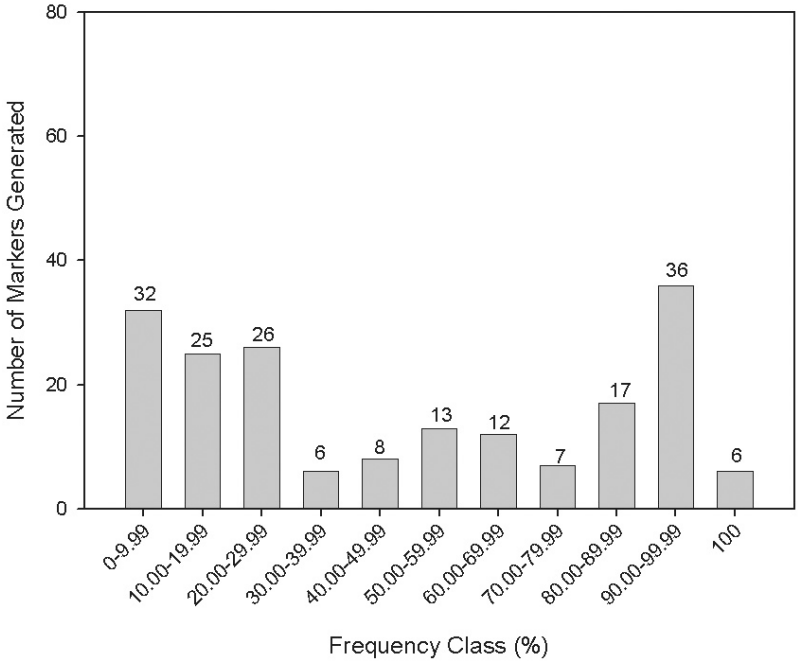

Figure 1. Number of sequence-related amplified polymorphism markers generated per frequency class, in percent, over all 189 bulked alfalfa DNA samples. Numbers above bars represent actual numbers of markers.

Table 3. Mean genetic similarity estimates (G_s) of entries based on the calculated Dice coefficient within and among populations. Underlined values represent within-population mean G_s, all others represent mean G_s between-populations.

Cultivar/ population†	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	26.	27.	28.	29.	30.	31.	32.	33.	34.	35.	36.	37.	38.
1. 5681	**0.90**																																					
2. Lobo	0.91	**0.91**																																				
3. Tahoe	0.87	0.87	**0.89**																																			
4. Wilson	0.78	0.77	0.76	**0.84**																																		
5. Amerigraze 701	0.77	0.77	0.78	0.71	**0.86**																																	
6. Arriba	0.77	0.78	0.79	0.70	0.83	**0.89**																																
7. Dona Ana	0.79	0.80	0.79	0.75	0.74	0.72	**0.90**																															
8. Dura 765	0.80	0.79	0.79	0.73	0.82	0.80	0.76	**0.87**																														
9. Malone	0.77	0.78	0.76	0.74	0.72	0.71	0.84	0.75	**0.87**																													
10. Rio	0.83	0.81	0.82	0.75	0.83	0.82	0.88	0.88	0.76	**0.91**																												
11. 13R Supreme	0.82	0.82	0.81	0.76	0.76	0.76	0.86	0.79	0.85	0.81	**0.92**																											
12. 58N57	0.82	0.82	0.82	0.76	0.76	0.75	0.80	0.80	0.78	0.82	0.83	**0.90**																										
13. Alto	0.82	0.81	0.82	0.75	0.76	0.74	0.80	0.79	0.76	0.72	0.81	0.85	**0.90**																									
14. Ameristand 801S	0.75	0.76	0.75	0.73	0.73	0.71	0.82	0.73	0.79	0.75	0.82	0.76	0.77	**0.81**																								
15. 5959	0.84	0.83	0.82	0.76	0.77	0.76	0.83	0.82	0.80	0.83	0.86	0.86	0.86	0.78	**0.91**																							
16. DK 180ML	0.81	0.81	0.82	0.75	0.78	0.77	0.79	0.78	0.75	0.80	0.80	0.82	0.84	0.76	0.85	**0.85**																						
17. DK 189	0.83	0.84	0.83	0.76	0.77	0.77	0.81	0.81	0.79	0.83	0.84	0.85	0.85	0.78	0.89	0.85	**0.89**																					
18. Magna 01FQ	0.80	0.80	0.80	0.74	0.79	0.78	0.77	0.77	0.75	0.80	0.80	0.82	0.80	0.77	0.84	0.83	0.84	**0.88**																				
19. RioGrande	0.81	0.81	0.80	0.75	0.78	0.75	0.80	0.80	0.77	0.74	0.82	0.86	0.86	0.76	0.87	0.83	0.86	0.82	**0.88**																			
20. 5929	0.77	0.74	0.75	0.71	0.73	0.71	0.73	0.77	0.73	0.79	0.76	0.78	0.75	0.70	0.78	0.76	0.79	0.75	0.77	**0.89**																		
21. 59N49	0.76	0.72	0.72	0.72	0.71	0.71	0.74	0.74	0.71	0.75	0.74	0.76	0.72	0.72	0.76	0.73	0.76	0.74	0.75	0.78	**0.84**																	
22. Beacon	0.76	0.76	0.75	0.73	0.72	0.72	0.72	0.77	0.73	0.77	0.76	0.75	0.75	0.71	0.80	0.76	0.78	0.72	0.78	0.80	0.80	**0.85**																
23. CUF 101	0.72	0.71	0.71	0.72	0.71	0.69	0.70	0.73	0.71	0.74	0.71	0.71	0.69	0.70	0.77	0.70	0.72	0.71	0.70	0.77	0.75	0.74	**0.87**															
24. DK 194	0.75	0.74	0.73	0.07	0.68	0.68	0.74	0.73	0.72	0.74	0.74	0.75	0.75	0.70	0.77	0.73	0.76	0.71	0.75	0.79	0.80	0.82	0.74	**0.84**														
25. Salado	0.77	0.76	0.74	0.71	0.71	0.69	0.75	0.75	0.72	0.73	0.74	0.77	0.76	0.72	0.78	0.74	0.86	0.73	0.77	0.79	0.78	0.81	0.74	0.81	**0.91**													
26. Sedona	0.77	0.75	0.75	0.71	0.73	0.71	0.77	0.77	0.72	0.73	0.75	0.82	0.75	0.72	0.77	0.75	0.76	0.73	0.76	0.81	0.80	0.79	0.79	0.79	0.94	**0.85**												
27. UC 1887	0.79	0.79	0.76	0.72	0.70	0.70	0.73	0.73	0.72	0.75	0.76	0.78	0.76	0.72	0.80	0.76	0.79	0.75	0.77	0.80	0.80	0.80	0.76	0.81	0.83	0.80	**0.91**											
28. WL 711	0.79	0.76	0.78	0.73	0.75	0.74	0.78	0.78	0.72	0.81	0.74	0.78	0.76	0.71	0.79	0.76	0.78	0.76	0.76	0.84	0.80	0.81	0.80	0.80	0.81	0.83	0.84	**0.91**										
29. UC 1465	0.77	0.76	0.74	0.70	0.68	0.69	0.73	0.73	0.71	0.74	0.75	0.78	0.75	0.70	0.78	0.75	0.76	0.73	0.76	0.81	0.81	0.79	0.74	0.81	0.79	0.77	0.84	0.80	**0.89**									
30. African	0.72	0.71	0.72	0.69	0.70	0.72	0.72	0.69	0.71	0.76	0.75	0.73	0.69	0.71	0.72	0.70	0.71	0.72	0.71	0.75	0.77	0.74	0.70	0.75	0.74	0.72	0.75	0.73	0.75	**0.85**								
31. Chilean	0.73	0.71	0.72	0.70	0.70	0.71	0.71	0.71	0.70	0.73	0.74	0.74	0.71	0.72	0.76	0.72	0.74	0.72	0.73	0.76	0.79	0.76	0.72	0.75	0.74	0.74	0.77	0.76	0.78	0.77	**0.88**							
32. Flemish	0.75	0.72	0.74	0.71	0.69	0.69	0.71	0.70	0.70	0.70	0.73	0.73	0.69	0.71	0.72	0.71	0.72	0.73	0.71	0.73	0.71	0.74	0.71	0.73	0.72	0.72	0.75	0.72	0.74	0.82	0.76	**0.86**						
33. Indian	0.72	0.72	0.70	0.68	0.65	0.66	0.69	0.68	0.70	0.69	0.72	0.72	0.70	0.68	0.72	0.71	0.73	0.69	0.72	0.74	0.74	0.75	0.70	0.73	0.72	0.71	0.77	0.74	0.73	0.75	0.74	0.74	**0.79**					
34. Ladak	0.71	0.70	0.68	0.71	0.67	0.67	0.69	0.70	0.69	0.70	0.70	0.70	0.68	0.70	0.71	0.68	0.71	0.71	0.70	0.74	0.77	0.73	0.74	0.73	0.72	0.74	0.76	0.75	0.75	0.73	0.75	0.76	0.70	**0.84**				
35. *M. falcata* WISFAL	0.70	0.70	0.70	0.66	0.68	0.66	0.67	0.65	0.66	0.66	0.67	0.69	0.67	0.67	0.66	0.66	0.67	0.80	0.67	0.67	0.70	0.68	0.66	0.68	0.72	0.67	0.72	0.68	0.69	0.76	0.71	0.75	0.68	0.69	**0.88**			
36. *M. varia*	0.74	0.73	0.72	0.70	0.70	0.71	0.69	0.71	0.69	0.72	0.72	0.75	0.71	0.70	0.74	0.71	0.74	0.68	0.73	0.75	0.79	0.75	0.71	0.76	0.76	0.74	0.79	0.76	0.77	0.75	0.78	0.73	0.71	0.76	0.73	**0.80**		
37. Peruvian	0.73	0.73	0.72	0.70	0.70	0.69	0.70	0.70	0.71	0.71	0.72	0.73	0.72	0.70	0.73	0.72	0.73	0.73	0.74	0.76	0.75	0.77	0.72	0.75	0.74	0.73	0.77	0.74	0.76	0.76	0.76	0.76	0.79	0.72	0.70	0.73	**0.86**	
38. Turkistan	0.74	0.74	0.71	0.72	0.68	0.74	0.70	0.70	0.73	0.71	0.74	0.74	0.71	0.73	0.76	0.68	0.68	0.70	0.74	0.77	0.76	0.76	0.71	0.61	0.73	0.73	0.76	0.74	0.75	0.73	0.73	0.74	0.77	0.72	0.68	0.72	0.78	**0.84**

†Cultivar/population column designations (1–38) correspond to cultivar/population row designation.

a population (Table 3), which ranged from 0.79 (Indian) to 0.88 (Chilean and *M. falcata* WISFAL) among the nine original sources of *Medicago* germplasm and from 0.81 to 0.92 among the 29 cultivars. The cultivar with the lowest mean G_s among bulks within a cultivar was Ameristand 801S (0.81), followed by two cultivars with mean $G_s = 0.84$ (Wilson and 59N49). The cultivar with the highest mean G_s among bulks within a cultivar was 13R Supreme (0.92) followed by six cultivars with mean $G_s = 0.91$ (Lobo, Rio, 5959, UC 1887 and Salado).

Estimating Genetic Similarities between Populations

The lowest mean G_s between populations representing the historical sources of *Medicago* germplasm was 0.68 between Turkistan and *M. falcata* WISFAL and between Indian and *M. falcata* WISFAL, and the highest was 0.82 between African and Flemish (Table 3). The lowest mean G_s between these sources of *Medicago* germplasm and alfalfa cultivars was 0.61 between Turkistan and DK 194, while the highest was 0.79 between UC 1887 and *M. varia* and between 59N49 and Chilean. The lowest mean G_s between alfalfa cultivars was 0.68 between Amerigraze 701 and DK 194, Amerigraze 701 and UC 1465, and Arriba and DK 194. The highest mean G_s between alfalfa cultivars was 0.94 between Sedona and Salado. The average of the mean G_s of a population determined for all combinations with other populations ranged from 0.71 (Wilson) to 0.79 (5959) for cultivars and from 0.68 (*M. falcata* WISFAL) to 0.74 (Chilean) for the original sources of *Medicago* germplasm. These results suggest that *M. falcata* WISFAL was the most genetically distinct source of *Medicago* germplasm included in this analysis, while Wilson was the most distinct cultivar.

Cluster analysis based on G_s revealed that the SRAP markers detected in bulked plant DNA samples could partially separate the populations examined in this study (Fig. 2: http://www.ars.usda.gov/sp2UserFiles/person/5791/PDF/alfalfadiversitydendro.pdf, verified 21 Aug. 2007). For the sake of narrative, some of the clusters have been numbered on the dendrogram and relevant subclusters have been labeled with letters. While there was a general lack of robust bootstrap values (>50%) in the base clusters, high support values were obtained for several subclusters joining different populations or bulk DNA samples of the same population.

Two clear clusters can be observed, one of which (#7) contains all five bulk samples of *M. falcata* WISFAL separated from the other bulks by a 100% bootstrap support value, and a large cluster composed of all the bulks of all cultivars and historical source populations except for *M. varia*, Bulk 5 and Indian, Bulk 1. This large cluster is initially separated into a single bulk of Ameristand and two major subclusters, one of which is further divided into subclusters 1 to 4, in which are located all of the bulks of cultivars in fall dormancy classes 6–8 (Table 1), and another that is further separated into subclusters 5 and 6, containing all the bulks of cultivars in fall dormancy classes 9–10 and the majority of the bulks of the nine populations representing the original sources of *Medicago* germplasm.

Subclusters on the dendrogram (Fig. 2) reveal additional grouping of cultivars that reflect similar fall dormancy classes (Table 1). Cluster 1 only contains bulks of cultivars in fall dormancy classes 7 and 8, Cluster 2A contains only bulks of fall dormancy class 6 cultivars, Cluster 2B only contains bulks of fall dormancy class 8 cultivars, Cluster 3 contains only bulks of fall dormancy class 7 cultivars, and Cluster 5 contains all the bulks of all the cultivars in fall dormancy classes 9 to 11 except for a single bulk of Beacon.

Several subclusters can be observed that are supported by robust bootstrap support values (>50) and only contain all the bulks of a single cultivar (Fig. 2). A subcluster containing all five bulks of 13R Supreme is observed in Cluster 1, while Cluster 4 is composed entirely of the five bulks of Wilson. All five bulks of cultivars 5929, UC 1465, UC 1887, Salado, and CUF 101 are located in Cluster 5 on individual subclusters supported by robust bootstrap support values.

Cluster 7 is composed of all five bulks of *M. falcata* WISFAL, while the bulks of the other historical sources of *Medicago* germplasm are primarily found in Clusters 5 and 6 (Fig. 2). Cluster 5 contains a subcluster supported by a robust bootstrap value that contains all five Chilean bulks. Between Clusters 5 and 6 is a well-supported cluster containing all five bulks of *M. varia*. Subcluster 6A contains all the Flemish and African bulks. Subcluster 6B separates into two additional subclusters, one of which contains all of the Peruvian bulks as well as four Indian bulks, and another, supported by high bootstrap values, containing all five Turkistan bulks.

To assess the importance of marker prevalence, cluster analyses were conducted using subsets of the marker data. Analysis of markers present in $\leq$90%, $\leq$60%, and 10 to 90% of the bulked DNAs did not provide better resolution of populations nor were better bootstrap support values obtained than what resulted from using all 188 markers (data not shown). The PCA showed the first three components to account for 11.9, 6.0, and 5.4% of variation within cultivars, and while additional components also accounted for significant variation based on the broken stick model (roughly 65% in total), they provided no further separation of populations.

DISCUSSION

In this report we describe the use of a marker strategy employing bulked DNA and SRAP markers to discriminating alfalfa populations. Previous marker analysis has suggested that *M. falcata* is the most distinct among populations representing the nine original sources of *Medicago*

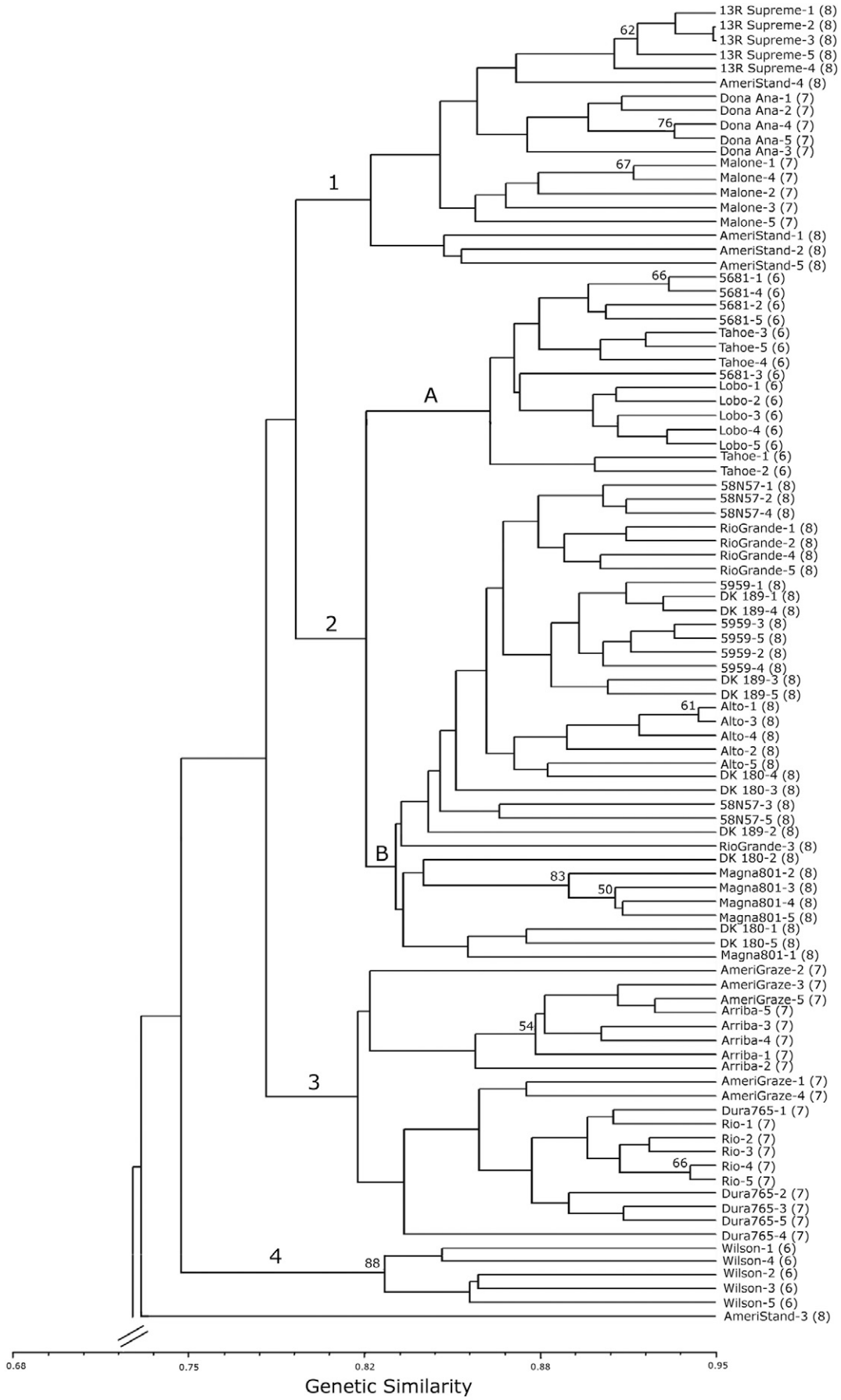

Figure 2. Part 1. Dendrogram showing results of cluster analysis of 189 bulked DNA alfalfa samples based on 188 sequence-related amplified polymorphism markers.

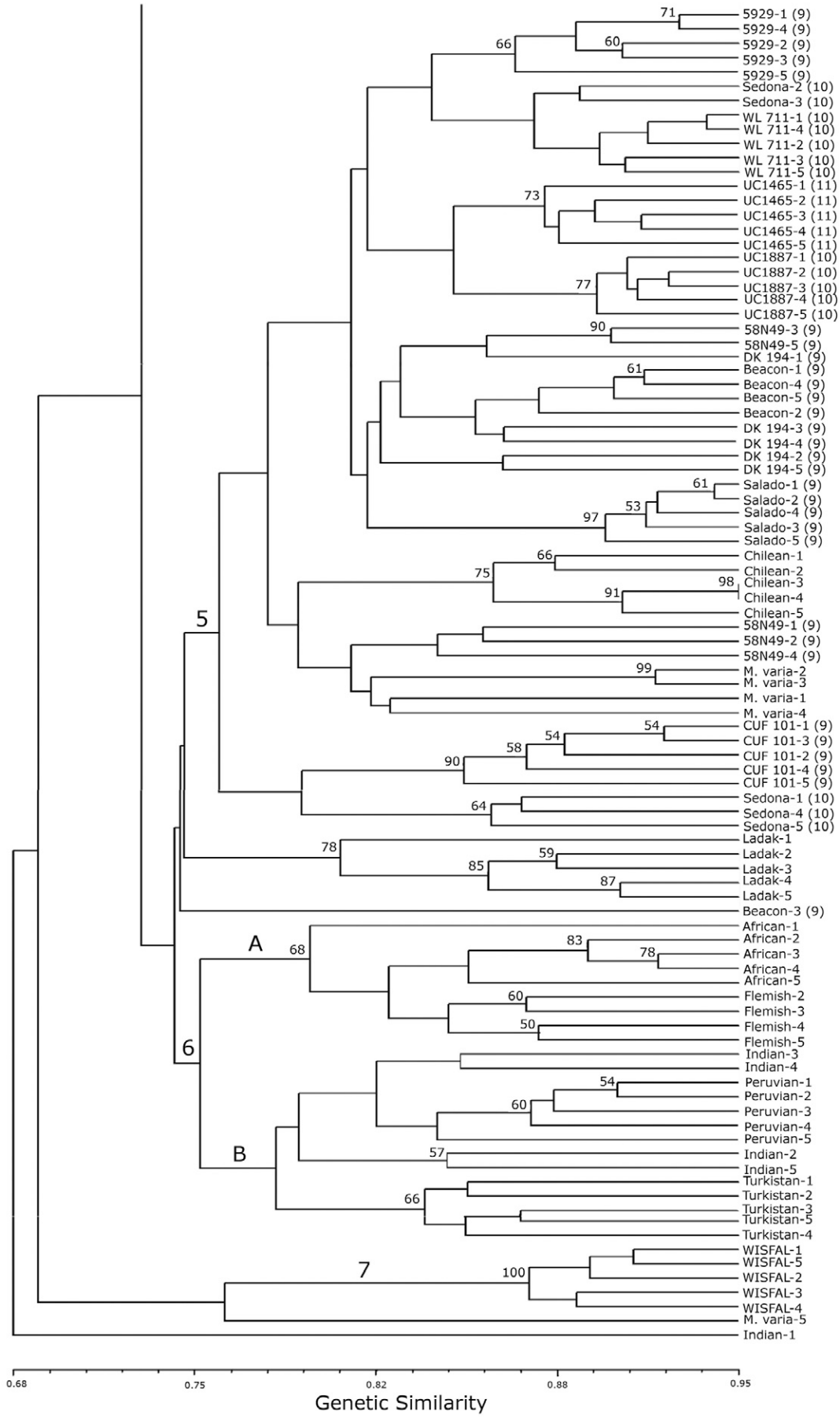

Figure 2. Part 2. Dendrogram showing results of cluster analysis of 189 bulked DNA alfalfa samples based on 188 sequence-related amplified polymorphism markers.

germplasm (Kidwell et al., 1994; Yu and Pauls, 1993; Segovia-Lerma et al., 2003; Vandemark et al., 2006). We observed similar results in this study, with cluster analysis separating all five bulks of *M. falcata* into a distinct cluster (Cluster 7) supported by high bootstrap values (Fig. 2).

A comparison of the dendrogram produced using SRAP data (Fig. 2) with that based on AFLP data (Segovia-Lerma et al., 2003) suggests that AFLPs were more effective than SRAPs at separated the nine germplasm sources into clusters that agree with expected relationships based on geographic origins (Segovia-Lerma et al., 2003). The AFLPs closely grouped the Chilean and Peruvian populations (Segovia-Lerma et al., 2003), whereas SRAPs grouped Chilean closer to *M. varia* than to Peruvian (Fig. 2). Segovia-Lerma et al. (2003) based their results on 3754 AFLPs, while our results are based on 188 SRAPs, and it is possible that an examination based on a greater number of SRAPs might produce results more similar to those reported by Segovia-Lerma et al. (2003). Different results based on SRAPs and AFLPs may also reflect differences in which populations were used to represent the original sources of germplasm. In this study we used the populations developed by Melton et al. (1990), whereas Segovia-Lerma et al. (2003) used older populations, which were later combined along with other populations by Melton et al. (1990). The populations developed by Melton et al. (1990) have a broader base of parental materials and are likely to be more genetically heterogeneous. In addition, we examined five bulks of 20 plants per bulk for each population, whereas Segovia-Lerma et al. (2003) examined a single bulk of 30 plants for each population, with each bulk being composed of 15 plants of each of two relic populations. Due to the reductive nature of cluster analysis, it is not surprising that a similar number of genotypes, bulked into a single categorical (i.e., population) sample, would produce tighter clusters on a dendrogram as compared to our design using five samples of genotype bulks per population category.

Examining multiple bulks for each population allowed for comparisons between populations in the mean G_s among bulks within a population. The within-population mean G_s of the Chilean and *M. falcata* WISFAL populations were the highest among the nine historical source populations, suggesting these two populations have the least genetic diversity. Kidwell et al. (1994) similarly observed that *M. falcata* WISFAL had the lowest within-population marker diversity among the nine germplasm sources based on RFLPs. The relatively high within-population mean G_s observed for the Chilean population may reflect a relatively narrow genetic base in the materials used to develop this population. The Chilean population was developed from intermating six populations, 'Chilean Common', 'Chilean 21-5', 'Chilean 21-5-5', 'California Common', 'California Common 49', and 'Caliverde' (Melton et al., 1990). All of these populations are estimated to be composed entirely of original introductions of Chilean germplasm into North America, except for Caliverde, which is estimated to contain 90% Chilean and 10% Turkistan germplasm (Barnes et al., 1977).

In contrast, the within-population mean G_s of the Indian and *M. varia* populations were the lowest among the nine historical source populations (Table 3). The detection of a relatively high amount of within-population marker diversity in *M. varia* is not surprising, since it was developed from crosses between PI 255178, which is an introduction from Poland, and populations derived from selections among hybrids between *M. falcata* and *M. sativa* that were independently introduced into North America from Germany and Russia (Barnes et al., 1977; Melton et al., 1990). The relatively high within-population marker diversity detected in Indian germplasm was not expected based on its pedigree (Melton et al., 1990), which indicates that it was developed from Arizona Indian, Sirsa No. 9 and Mesa-Sirsa, all of which are considered to be composed entirely from the original introduction of Indian germplasm into California. However, Warburton and Smith (1993) examined agronomic and morphological traits in 21 different PI accessions from northern and western India and found that cluster analysis separated the accessions into four clusters. The relatively low within-population mean G_s of the Indian population may be partially due to the effects of sampling a finite number of markers, but may also reflect greater genetic variation in this population than would be expected based on the classification system proposed by Barnes et al. (1977).

Although in general, all five bulks of a population tended to cluster closely together on the dendrogram (Fig. 2), there were several individual bulks, most notably Indian 1, *M. varia* 5, Beacon 3, and AmeriStand 3 that were distant on the dendrogram from the four other bulks of each respective population. The mean within-population G_s of Indian 1, *M. varia* 5, Beacon 3, and AmeriStand 3 were 0.75, 0.76, 0.79, and 0.78, respectively, while the average mean within-population G_s of the other four bulks of each population were 0.80, 0.81, 0.87, and 0.82. This indicates that each distant bulk had a mean within-population G_s that was at least 90% of the average mean within-population G_s of the other four bulks of its population. The magnitude of these differences would likely be greater if they were the result of gross technical errors in PCR, such as a lack of amplification in these distant bulks, or the spurious amplification of a large set of unique SRAPs in these bulks. In addition, the amplification profiles generated using these four distant bulks and several different primer pairs were unanimously reproduced using two different thermocyclers. These observations suggest that the positioning of these distant bulks on the dendrogram may be

reflecting differences in allele frequencies between these bulks and other bulks of the same populations.

Cultivars in FDC 9 to 11 were more closely grouped with the populations representing the original sources of *Medicago* germplasm than were the cultivars in FDC 6 to 8 (Fig. 2). This suggests that the very nondormant cultivars (FDC 9–11) examined in this study are more similar genetically to the original sources of alfalfa germplasm than are cultivars in FDC 6 to 8. Cultivars in FDC 6 to 8 typically have higher levels of resistance than cultivars in FDC 9 to 11 to several diseases, including bacterial wilt, anthracnose, and stem nematodes (Alfalfa Council, 2005). The closer association of the very nondormant cultivars to the original *Medicago* germplasm sources may reflect greater genetic divergence of the FDC 6 to 8 cultivars from the original source populations as a consequence of selection for traits such a resistance to multiple diseases. The distant grouping of the majority of cultivars from the original sources of Medicago germplasm (Fig. 2) suggests that the original sources of alfalfa germplasm may only have limited utility for improving alfalfa cultivars.

Several alfalfa cultivars, including Wilson and 13R Supreme, could be distinguished based on cluster analysis of SRAP marker data (Fig. 2). An averaging of the mean G_s across all entries (Table 3) indicated that Wilson was the most genetically distinct cultivar. The cultivar description for Wilson suggests that it is unique among the other cultivars examined in this report in that it is constituted primarily of Turkistan (72%) and Chilean (22%) parentage. 13R Supreme also has a rather unique combination of contributions from the historical sources of germplasm among the cultivars examined, in that it is estimated to be composed of 70% African and 30% Turkistan germplasm.

Some relationships were observed between cultivars based on cluster analysis that agreed with similarities in reported pedigrees (Fig. 2). For example, 5929 was found in close proximity to CUF 101, which was one of the parental populations used in the development of 5929 (Lehman et al., 1983; Woodward et al., 1988). Very close clustering was also observed between Malone and Dona Ana, which was one of two parental populations used in the development of Malone (Melton et al., 1989). These results are encouraging with respect to the ability of SRAP markers to suggest genetic relationships that reflect pedigree information. However, there was no close grouping of Ameristand 801S bulks with bulks of Salado, which is the cultivar from which Ameristand 801S was selected directly. Similarly, Arriba was largely selected from Lobo, but these two cultivars did not closely group together. Several factors may have contributed to discrepancies between clustering based on SRAP markers and expected relationships based on pedigree information. These include the number of SRAP markers detected, possible changes in gene frequencies between newer cultivars and earlier cultivars due to random mating among selected parental clones and progeny, genetic shift during seed production, and directed selection for desirable traits in the newer cultivars.

In a preliminary analysis of alfalfa cultivars with SRAPs, clustering among cultivars was observed that reflected shared fall dormancy ratings, with the dormant cultivars Oneida (FDC = 2) and Vernal (FDC = 3) clustering together while the less dormant cultivars Malone (FDC = 7) and CUF101 (FDC = 9) also formed a distinct cluster (Vandemark et al., 2006). Similar results were observed in this study (Fig. 2). In addition, a SRAP marker was identified that was present only in bulks of FDCs 6 and 7, and another marker was identified that was detected only in bulks of FDC 8. The process of evaluating plants and populations for fall dormancy would be greatly accelerated if a molecular marker could be identified that was associated with the this trait. It will be a considerable challenge to develop a set of markers that can discriminate among all FDCs. Nonetheless, the overall separation of cultivars based on cluster analysis and the identification of SRAPs associated with specific fall dormancy classes indicates that these markers have potential for accurately discriminating alfalfa cultivars based on fall dormancy.

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